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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 5/00, 15/00, C12P 21/00

A1

(11) International Publication Number: WO 90/03431

(43) International Publication Date: 5 April 1990 (05.04.90)

(21) International Application Number: PCT/US89/04164

(22) International Filing Date: 25 September 1989 (25.09.89)

(30) Priority data:

249,446	26 September 1988 (26.09.88)	US
265,446	1 November 1988 (01.11.88)	US
311,517	13 February 1989 (13.02.89)	US
323,964	15 March 1989 (15.03.89)	US

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.

(54) Title: MIXED FEED RECOMBINANT YEAST FERMENTATION

(57) Abstract

The invention provides a method of increasing the production of a recombinant gene product from a culture of a recombinant methylotrophic yeast host, wherein said product is made by expression from a recombinant gene sequence operably associated with a methanol-responsive expression control element. In the method of the invention, the methylotrophic yeast host is first cultured on a medium with a high concentration of multi-carbon, carbon-source nutriment, such as glycerol, but with little or no methanol, in order to increase the density of the host cells with little or no expression of the recombinant gene product. When the host cells have achieved a suitable density in the culture medium, the culture is subjected to a phase during which the concentration of multi-carbon, carbon-source nutriments is maintained sufficiently low that the methanol-responsive control element controlling expression of the recombinant gene encoding the desired product is derepressed. Finally, the culture is subjected to a phase of high production of the recombinant gene product by increasing the concentration of methanol while maintaining the concentration of multi-carbon, carbon-source nutriments at a low level. The invention is illustrated with production, using *Pichia pastoris*, of bovine lysozyme c2, human lysozyme, human epidermal growth factors (1-52) and (1-48), and human superoxide dismutase.

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MIXED FEED RECOMBINANT YEAST FERMENTATION

Field of the Invention

This invention relates generally to refinements in fermentation technology, particularly directed to improvements for recombinant yeast production, allowing for the preparation of enhanced amounts of heterologous protein produced in recombinant yeast host strains by expression of a heterologous gene encoding said protein in a controlled, high-production manner. The present invention thus utilizes refinements in the growth and culturing techniques of a given recombinantly harnessed yeast host to allow production of recombinant protein in high cell density fermentation so as to maximize recovery of heterologous gene product. The present invention is especially suited for use with _Pichia (P.) pastoris_ yeast hosts, although any yeast host having equivalent culturing properties and susceptibilities, such as, for example, _Hansenula polymorpha_, may also be utilized with success in accordance with the teachings hereof.

Background of the Invention

Yeast host strains have proved particularly adaptable for use in recombinant host systems wherein heterologous (to the yeast host) gene expression is employed in large scale fermentation to produce large amounts of biologically useful heterologous gene product, that is, heterologous polypeptides. These yeast hosts are particularly suitable in recombinant systems because they seem to combine the advantages of both prokaryotes and eukaryotes, and yet provide easily manipulatable characteristics such that maximum exploitation in a recombinant fermentation setting can be explored. For example, similar to prokaryotes, yeasts are unicellular organisms that have relatively rapid growth rates and are relatively easily transformed with heterologous DNA cassettes having

operably arranged control elements for a given heterologous gene. By the same token, yeasts provide the advantages ordinarily thought unique to higher eukaryotes, such as an apparatus for incorporating glycosylation structure and, in frequent cases, utilization of secretory pathways such that the heterologous gene product can be more easily recovered from the support culture medium of the host yeast organism.

Much attention has focused recently on the P. pastoris species of yeast hosts. This methylotrophic yeast has been shown to be an outstanding host for high-level heterologous gene expression. In major part, the overall success of the Pichia expression system is tied to the strength of the promoter of its major alcohol oxidase (AOX1) gene, said strength demonstrated by observations that the alcohol oxidase enzyme comprises up to 30% of the protein in extracts of native P. pastoris when grown on methanol. The AOX1 gene promoter has been isolated, cloned and transformed into P. pastoris strains from as early as 1985. Another key feature of this methylotrophic yeast host is that high cell densities can be economically achieved in a simple methanol-nutriment-salts medium. In such an environment, Pichia has been demonstrated to be capable of producing high levels of heterologous gene products, including hepatitis B virus surface antigen, invertase, animal lysozyme c's, and others. Some of these heterologous gene products have the additional advantage of being recognized by the Pichia secretory processes such that the products are secreted into the medium supporting the host cells and can be isolated from that medium.

The first report of a yeast which could utilize methanol for its growth did not appear until the late 1960s. Since then, only a few species of methylotrophic yeasts have been identified. They exist in four genera, namely, Candida, Hansenula, Pichia and

Torulopsis. The biochemical pathway for methanol utilization in these genera has been defined and appears to be similar. The term "methylotrophic" indicates a yeast's ability to grow on methanol, among other carbon source nutriments. Researchers have found that these strains can utilize simultaneously methanol and glucose mixtures or other mixed carbon sources such as methanol with formate, glycerol, ribose, sorbitol, or xylose nutriments. See Gleeson *et al.*, *Yeast* *4*, 1 (1988).

Research also has been conducted on a particular genus of yeast, namely *Hansenula*, and, in particular, the methylotrophic species *Hansenula polymorpha*. This research has focused on mechanistic studies of support media for enhancing growth rate and has involved studies of carbon utilization from medium of different compositions by the species in a carbon-limited chemostat culture system. It was found that, with all of the mixtures tested, a similar utilization pattern evolved: At low dilution rates, both methanol and higher carbon sources were utilized simultaneously; but, at the higher dilution rates, the cells preferentially used the higher carbon sources (*i.e.*, compounds with more than one carbon) while, for the most part, unutilized methanol accumulated in the culture medium. See Egli, *et al.*, *Biotechnology and Bioengineering* *28*, 1735 (1986), and various references cited therein for reports on similar research.

Researchers in the same laboratories found that, when mixtures of carbon sources (C_1 and C_6) containing higher proportions of methanol were used, regular growth yields for methanol were recorded which corresponded to the growth yields found with methanol when it was used as the only carbon source. Further, these researchers found that, for example, glucose, when present in high levels in the medium, repressed the methanol metabolic pathway. When glucose was

present in limiting concentrations, derepression of the methanol pathway was observed. Where methanol concentrations were increased in these latter systems, induction by methanol of the methanol metabolic pathway was observed. This degree of induction was found to increase with increasing proportions of methanol in the mixed substrate mixture. See Egli et al., Archives of Microbiology 131, 1 (1982). Attention is also directed to various other prior work such as Egli, et al., Microbial Growth on C1 Compounds; Crawford, et al., eds., American Society for Microbiology, Washington, D.C. (1984); Egli, et al., Journal of General Microbiology 132, 1779 (1986); Eggeling, et al., Archives of Microbiology 127, 119 (1980); Eggeling, et al., Archives of Microbiology 130, 362 (1981); Hazeu, et al., Biotech Letters 5, 399 (1983); Mueller, et al., Appl. Microb. Biotech. 25, 238 (1986); Swartz et al., Appl. Envir. Microbiol. 41, 1206 (1981).

All of these prior researchers worked in chemostat systems, thereby causing in a controlled study the changing of the concentration of a particular nutriment in the overall culture medium. Further, these researchers used cultures of non-recombinant organisms and cultures at relatively low densities in their studies. Hence, these researchers experimented with systems wherein the host yeast organisms were not susceptible to genetic load pressures to mutate or otherwise eliminate the foreign gene and its functional product and wherein problems were not presented by the irregularities known to exist when high culture densities are employed so as to exploit maximally the cell culture volume.

Further, these researchers avoided other problems associated with high density fermentation. Specifically, high cell density fermentations are often sustained by the supply of high substrate feed concentrations and/or high rates of feed addition.

Both of these parameters are known to influence yeast physiology to the extent that ethanol, a by-product of multi-carbon substrate metabolism, can accumulate in the growth medium. This can lead to lower product yields since ethanol is a potent repressor of the alcohol oxidase promoter.

In contrast, research with recombinant yeast systems demands high-density, highly exploitive culture manipulation techniques so as to maximize production levels of heterologous products for isolation and recovery of usable amounts in the biotechnology industry. These host cells are not only subjected to extremes in culturing techniques, making them susceptible to variability in growth patterns caused from the intense culturing refinements, but also must tolerate their production of copious amounts of heterologous gene products which are historic new-comers to their environment. The biological response for these hosts is to eliminate production of the heterologous protein by induced mutation or otherwise so as to rid itself of the foreign gene and its product.

Further, high density culturing introduces the added problem of increased ethanol by-product formation that interferes with the efficiency of transcription from the alcohol oxidase promoter.

Hence, results that may be recorded by prior researchers in respect of relatively low density, nonrecombinant, wild-type yeast host culture chemostat systems have no bearing on research efforts using distinct and different recombinant systems. Because of this, the art affords no basis upon which to suggest results when employing, for example, high yielding manipulative culturing techniques in a recombinant host system.

Summary of the Invention

The present invention is based upon the unpredictable results that enhanced yield over time (throughput) of recombinant protein can be obtained from cultured recombinant methylotrophic yeast hosts using a mixed-nutriment-feed, cell growth-gene induction mode of culturing comprising:

(1) a high growth phase wherein the nutriment medium contains a high concentration of multi-carbon carbon-source nutriment with little or no methanol, for a period of time sufficient to increase the density of the viable yeast cells in the growth medium, without their producing by expression any substantial amount of recombinant gene product,

(2) feeding a limiting amount of multi-carbon, carbon-source nutriment for a period of time sufficient to derepress the methanol metabolic pathway of said yeast host,

(3) allowing the fermentation culture to be maintained in a phase of high production of recombinant gene product during which time the methanol concentration is increased while maintaining a low multi-carbon, carbon-source nutriment.

Thus, the present invention is predicated upon a method of increasing the production of recombinant gene product from a culture of recombinant methylotrophic yeast hosts, wherein said recombinant gene product is made by expression of a recombinant gene sequence operably associated with a methanol-responsive expression control element, which method comprises culturing said methylotrophic yeast host using a mixed nutriment feed, cell growth-gene induction mode comprising:

(a) a high growth phase wherein the nutriment medium contains a high concentration of multi-carbon, carbon-source nutriment with little or no methanol, for a period of time sufficient to

increase the density of the viable yeast cells in the growth medium, without their producing by expression any substantial amount of said recombinant gene product,

(b) feeding a limiting amount of multi-carbon, carbon-source nutriment for a period of time sufficient to derepress the methanol metabolic pathway of said yeast host, and

(c) allowing the fermentation culture to be maintained in a phase of high production of recombinant gene product during which time the methanol concentration is increased while maintaining a low multi-carbon, carbon-source nutriment.

As understood in the art of making recombinant gene products by culturing microorganisms engineered to make the products, a product made in accordance with the invention is usually recovered and purified from the yeast culture, although in some cases cell-free culture medium comprising the recombinant gene product can be used without separation of the product from the medium, in some cases cells or parts of cells (e.g., the membrane fraction thereof) comprising the recombinant gene product can be used without separation of the product from the cells or parts thereof, and in some cases the yeast culture as such, including both cells and medium and comprising the recombinant gene product in either or both, can be used directly. Recovery and purification of recombinant gene product from a yeast culture can be accomplished by any of numerous methods well known to the skilled.

Recombinant methylotrophic yeast hosts, on which the method of the invention is practiced to improve the production of recombinant gene product, are known in the art or can be obtained by known methods comprising transforming a methylotrophic yeast with recombinant DNA comprising a gene sequence encoding the

recombinant gene product and a methanol-responsive expression control element, operably associated for expression with the sequence encoding the recombinant gene product, and selecting the yeast so transformed.

Brief Description of the Drawings

Figure 1 is a plot showing concentration of bovine lysozyme c2 with time in the induction phase in 1 liter fermentations of the Mut^- (methanol utilization-deficient) strain grown on methanol alone (open squares), 4:1 glycerol to methanol mixture (closed squares), 2:1 glycerol to methanol mixture (closed diamonds), or a limiting glycerol/non-limiting (NL) methanol mixture (open diamonds).

Figure 2 is a plot showing concentration of bovine lysozyme c2 with time in the induction phase in 1 liter fermentations of the Mut^- strain grown on methanol alone (open diamonds), a limiting glycerol/ non-limiting (NL) methanol mixture (open squares), or the Mut^+ strain grown under the limited methanol protocol (closed squares).

Figure 3A is a plot showing concentration of bovine lysozyme c2 with time in the induction phase of the Mut^- strain grown on methanol alone in a 1 liter fermentor (open squares) and a 10 liter fermentor (closed squares).

Figure 3B is a plot showing concentration of lysozyme with time in the induction phase of the Mut^- strain grown on a mixed glycerol and methanol feed in a 1 liter fermentor (open squares) and a 10 liter fermentor (closed squares).

Detailed Description of the Invention

The present invention is described with more particularity featuring, as a model system, the production of bovine lysozyme c2 under the control of the major alcohol oxidase (AOX1) gene promoter in

recombinant Pichia pastoris yeast hosts. It is understood that the invention is equivalently practiced using any one of other methylotrophic yeast hosts which harbor, on autonomously replicating plasmids or episomes or in the genome, DNA encoding any of a wide variety of desired, recombinant products (e.g., polypeptides heterologous to the methylotrophic yeast host), which DNA is expressed under the regulation of a methanol-responsive control element. Among these desired products are human lysozyme, human superoxide dismutase, and human epidermal growth factor (EGF)(1 - 52) and (1 - 48).

In its preferred embodiments, the present invention of a mixed-feed fermentation mode employs glycerol as a carbon source for growth and methanol as a carbon source for both growth and induction of heterologous gene expression. The two feeds work in combination to allow more efficient cell growth and heterologous gene expression.

The mixed-feed mode of fermentation is an advantageous way of growing both Mut^+ and Mut^- strains, which have an intact and defective AOX1 gene, respectively. Because they lack a functional AOX1 gene, Mut^- strains grow much more slowly on methanol than Mut^+ strains do. This characteristic necessitates conducting fermentations of recombinant Mut^- strains, when methanol is the carbon source, for considerably longer times than that required for corresponding Mut^+ strains. One advantage gained by the mixed-feed fermentation mode is that it allows the Mut^- strain to grow and synthesize product in a more time-efficient manner, thus substantially increasing volumetric productivity. The volumetric productivity of the Mut^+ and Mut^- strains can also be increased with the use of mixed feeds in continuous fermentations.

For Mut^+ and Mut^- strains, the mixed-feed mode of fermentation is advantageous because it decreases

the heat load and oxygen demand on the fermentation, while at the same time maintaining a relatively high level of heterologous gene expression. Because most fermentors have a limited capacity for heat and oxygen transfer, and because cell density and productivity are related to a fermentation vessel's heat and oxygen transfer capacity, any decrease in the organism's cooling and oxygen demand should result in an enhanced capacity for cellular productivity. The strains grown in the invented mode also grow faster on a mixed feed than they do on methanol alone.

The mixed-feed mode of cell growth and gene induction is operable in both continuous and fed batch fermentations.

The protocols for Mut^- and Mut^+ cell growth in mixed feed fermentations are described briefly below.

The fermentations are run in three broadly defined stages:

First, the fermentor containing medium with 4% or higher content of glycerol is inoculated with cells that have been grown in YNB (yeast nitrogen base) plus 2% glycerol. The AOX1 promoter is repressed under these conditions so recombinant product is not made. The cells' energy is instead directed toward increasing the cell density.

After the cell density reaches the desired level upon depletion of glycerol, cell densities are about 35 g/l (dry wt) in a 14L vessel, and 20 g/l in a 2L vessel, the cells are started on a glycerol-limited fed batch mode of fermentation. This glycerol feed lasts for about 4 to 12 hours, most commonly about 4 to 6 hours, and allows the AOX1 promoter to gradually shift from a repressed to a fully derepressed stage.

The mixed-feed phase of the fed-batch fermentation, which usually starts at about 30 hours and continues preferably for up to about 40 hours more,

is initiated by starting a methanol feed, for example, at a 4:1 or 2:1 (glycerol:MeOH) ratio. Both the glycerol and methanol feed contain 12 ml/L of YTM_4 and IM_1 trace metals.

In one preferred mode for Mut^- cells, a 2:1 ratio of glycerol and methanol is fed into the fermentor for approximately eight hours after the transition phase to ensure that the cells are growing normally and the AOX1 promoter is fully derepressed. Then, the methanol feed rate is increased to give residual methanol concentration between 0.1-0.8%, while the glycerol feed stays constant.

After the mixed-feed phase of the fed-batch fermentation, a continuous fermentation can be initiated by starting a basal salts feed. Whole broth and cells are removed from the fermentor at the rate at which MeOH, glycerol, and basal salts are added; this keeps the fermentor volume constant. (The effluent flow rate divided by the fermentor volume gives a characteristic value known as the dilution rate.)

The mixed-feed modes consistently allow higher productivity in Mut^- strains, as compared to growth in a methanol excess mode. In Mut^+ strains, the mixed feed mode is equally, if not more so, productive as the other methods of growth.

EXAMPLES

1. Bovine Lysozyme c2

Strain

The cDNA encoding bovine lysozyme c2 (the most abundant lysozyme of the bovine abomasum) was isolated from a cDNA library prepared in gt10 using poly A+ RNA isolated from bovine abomasum tissue. The library was screened with a partial genomic clone, pL1, which comprises exons 2 and 3 and a portion of exon 4

of the bovine lysozyme c2 gene. Positive clones were confirmed by screening with an oligonucleotide complementary to the 5'-end of exon 2. Sequencing of the insert of one of the clones, λBL3, revealed that the sequence encoded a full length coding sequence for the bovine lysozyme, but lacked an initiator methionine, indicative of only a partial signal sequence. The missing signal sequence was added to the insert using site-directed mutagenesis and the sequence of the missing nucleotides obtained by direct sequencing of abomasum mRNA for the preprotein. Mutagenesis was also used to add EcoRI sites to the 5' and 3' ends of the insert, which now encoded the full signal sequence and mature protein for bovine lysozyme c2. The EcoRI fragment was inserted into EcoRI-cut Pichia expression plasmid pAO804 (construction described below).

Plasmid pAO804 is comprised of sequences from the 5' and 3' ends of the P. pastoris AOX1 gene which are used to target the BglII-cut vector to insert at and disrupt the AOX1 locus. The plasmid additionally comprises the P. pastoris AOX1 transcription promoter and terminator, the P. pastoris HIS4 gene, and sequences necessary for replication and selection in bacteria. A unique EcoRI site separates the promoter and terminator regions, enabling the insertion of heterologous genes at this site.

Plasmid pAO804 was constructed as follows:

pBR322 was digested with EcoRI, the protruding ends were filled in with Klenow Fragment of E. coli DNA polymerase I, and the resulting DNA was recircularized using T4 ligase. The recircularized DNA was used to transform E. coli MC1061 to ampicillin-resistance and transformants were screened for having a plasmid of about 4.37 kbp in size without an EcoRI site. One such transformant was selected and cultured

to yield a plasmid, designated pBR322△RI, which is pBR322 with the EcoRI site replaced with the sequence:

5'-GAATTAATTC-3'

3'-CTTAATTAAG-5'

pBR322△RI was digested with PvuII and the linker, of sequence

5'-CAGATCTG-3'

3'-GTCTAGAC-5'

was ligated to the resulting blunt ends employing T4 ligase. The resulting DNAs were recircularized, also with T4 ligase, and then digested with BglII and again recircularized using T4 ligase to eliminate multiple BglII sites due to ligation of more than one linker to the PvuII-cleaved pBR322△RI. The DNAs, treated to eliminate multiple BglII sites, were used to transform _E. coli_ MC1061 to ampicillin-resistance. Transformants were screened for a plasmid of about 4.38 kbp with a BglII site. One such transformant was selected and cultured to yield a plasmid, designated pBR322△RIBGL, for further work. Plasmid pBR322△RIBGL is the same as pBR322△RI except that pBR322△RIBGL has the sequence

5'-CAGCAGATCTGCTG-3'

3'-GTCGTCTAGACGAC-5'

in place of the PvuII site in pBR322△RI.

pBR322△RIBGL was digested with SalI and BglII and the large fragment (approximately 2.97 kbp) was isolated. Plasmid pBSAGI5I, which is described in European Patent Application Publication No. 0 226 752, was digested completely with BglII and XhoI and an approximately 850 bp fragment from a region of the _P. pastoris_ AOX1 locus downstream from the AOX1 gene transcription terminator (relative to the direction of transcription from the AOX1 promoter) was isolated. The BglII-XhoI fragment from pBSAGI5I and the approximately 2.97 kbp, SalI-BglII fragment from pBR322△RIBGL were combined and subjected to ligation with T4 ligase. The ligation mixture was used to

transform E. coli MC1061 to ampicillin-resistance and transformants were screened for a plasmid of the expected size (approximately 3.8 kbp) with a BglII site. This plasmid was designated pAO801. The overhanging end of the SalI site from the pBR322/$\triangle$RIBGL fragment was ligated to the overhanging end of the XhoI site on the 850 bp pBSAGI5I fragment and, in the process, both the SalI site and the XhoI site in pAO801 were eliminated.

pBSAGI5I was then digested with ClaI and the approximately 2.0 kbp fragment was isolated. The 2.0 kbp fragment has an approximately 1.0 kbp segment which comprises the P. pastoris AOX1 promoter and transcription initiation site, an approximately 700 bp segment encoding the hepatitis B virus surface antigen ("HBsAg") and an approximately 300 bp segment which comprises the P. pastoris AOX1 gene polyadenylation signal and site-encoding segments and transcription terminator. The HBsAg coding segment of the 2.0 kbp fragment is terminated, at the end adjacent the 1.0 kbp segment with the AOX1 promoter, with an EcoRI site and, at the end adjacent the 300 bp segment with the AOX1 transcription terminator, with a StuI site, and has its subsegment which codes for HBsAg oriented and positioned, with respect to the 1.0 kbp promoter-containing and 300 bp transcription terminator-containing segments, operatively for expression of the HBsAg upon transcription from the AOX1 promoter. The EcoRI site joining the promoter segment to the HBsAg coding segment occurs just upstream (with respect to the direction of transcription from the AOX1 promoter) from the translation initiation signal-encoding triplet of the AOX1 promoter.

For more details on the promoter and terminator segments of the 2.0 kbp, ClaI-site-terminated fragment of pBSAGI5I, see European Patent

Application Publication No. 0 226 846 and Ellis et al., Mol. Cell. Biol. 5, 1111 (1985).

Plasmid pAO801 was cut with ClaI and combined for ligation using T4 ligase with the approximately 2.0 kbp ClaI-site-terminated fragment from pBSAGI5I. The ligation mixture was used to transform E. coli MC1061 to ampicillin resistance, and transformants were screened for a plasmid of the expected size (approximately 5.8 kbp) which, on digestion with ClaI and BglII, yielded fragments of about 2.32 kbp (with the origin of replication and ampicillin-resistance gene from pBR322) and about 1.9 kbp, 1.48 kbp, and 100 bp. On digestion with BglII and EcoRI, the plasmid yielded an approximately 2.48 kbp fragment with the 300 bp terminator segment from the AOX1 gene and the HBsAg coding segment, a fragment of about 900 bp containing the segment from upstream of the AOX1 protein encoding segment of the AOX1 gene in the AOX1 locus, and a fragment of about 2.42 kbp containing the origin of replication and ampicillin resistance gene from pBR322 and an approximately 100 bp ClaI-BglII segment of the AOX1 locus (further upstream from the AOX1-encoding segment than the first mentioned 900 bp EcoRI-BglII segment). Such a plasmid had the ClaI fragment from pBSAGI5I in the desired orientation; in the opposite undesired orientation, there would be EcoRI-BglII fragments of about 3.3 kbp, 2.38 kbp and 900 bp.

One of the transformants harboring the desired plasmid, designated pAO802, was selected for further work and was cultured to yield that plasmid. The desired orientation of the ClaI fragment from pBSAGI5I in pAO802 had the AOX1 gene in the AOX1 locus oriented correctly to lead to the correct integration into the P. pastoris genome at the AOX1 locus of linearized plasmid made by cutting at the BglII site at the terminus of the 800 bp fragment from downstream of the AOX1 gene in the AOX1 locus.

pAO802 was then treated to remove the HBsAg coding segment terminated with an EcoRI site and a StuI site. The plasmid was digested with StuI and a linker of sequence:

5'-GGAATTCC-3'
3'-CCTTAAGG-5'

was ligated to the blunt ends using T4 ligase. The mixture was then treated with EcoRI and again subjected to ligation using T4 ligase. The ligation mixture was then used to transform E. coli MC1061 to ampicillin resistance and transformants were screened for a plasmid of the expected size (5.1 kbp) with EcoRI-BglII fragments of about 1.78 kbp, 900 bp, and 2.42 kbp and BglII-ClaI fragments of about 100 bp, 2.32 kbp, 1.48 kbp, and 1.2 kbp. This plasmid was designated pAO803. A transformant with the desired plasmid was selected for further work and was cultured to yield pAO803.

Plasmid pAO804 was then made from pAO803 by inserting, into the BamHI site from pBR322 in pAO803, an approximately 2.75 kbp BglII fragment from the P. pastoris genome which harbors the P. pastoris HIS4 gene. See, e.g., Cregg et al., Mol. Cell. Biol. 5, 3376 (1985) and European Patent Application Publication Nos. 0 180 899 and 0 188 677. pAO803 was digested with BamHI and combined with the HIS4 gene-containing BglII site-terminated fragment and the mixture subjected to ligation using T4 ligase. The ligation mixture was used to transform E. coli MC1061 to ampicillin-resistance and transformants were screened for a plasmid of the expected size (7.85 kbp), which is cut by SalI. One such transformant was selected for further work, and the plasmid it harbors was designated pAO804.

pAO804 has one SalI-ClaI fragment of about 1.5 kbp and another of about 5.0 kbp and a ClaI-ClaI fragment of 1.3 kbp; this indicates that the direction of transcription of the HIS4 gene in the plasmid is the

same as the direction of transcription of the ampicillin resistance gene and opposite the direction of transcription from the AOX1 promoter.

The orientation of the HIS4 gene in pAO804 is not critical to the function of the plasmid or of its derivatives with cDNA coding segments inserted at the EcoRI site between the AOX1 promoter and terminator segments.

The _Pichia pastoris_ strain GS115 (_his4_) - Cregg et al; _Molecular and Cellular Biology_ _15_, 3376 (1985), ATCC No. 20864 - was used as the host strain for transformation with restriction fragments of the bovine lysozyme expression plasmid pSL12A; see Digan _et al._, _Dev. Industr. Microbiol._ _29_, 59 (1988).

The final expression vector, which contains the bovine lysozyme gene, vector pSL12A, was used to develop both Mut^+ and Mut^- transformants.

"Mut" = methanol utilization. In Mut^+ strains, growth on methanol is similar to that of wild-type strains, because the AOX1 gene remains functional. In Mut^- strains, growth on methanol still occurs, because of the presence in _P. pastoris_ of a minor alcohol oxidase gene (AOX2 gene) which remains functional, but the growth is significantly slower than that of Mut^+ strains on methanol.

pSL12A was digested with BglII and the BglII fragment was transformed into GS115 cells using the whole cell lithium chloride transformation system (Cregg _et al._, _Molecular and Cellular Biology_, _5_, 3376 (1985)). Mut^- cells were recovered from this transformation. A representative Mut^- strain was called A37.

The Mut^+ transformants were developed by digesting pSL12A with SalI, which cuts the plasmid once in the _HIS4_ gene, and using the SalI digested plasmid in the whole cell transformation protocol. Mut^+ cells were recovered from this transformation. A representative Mut^+ strain was called L1.

The strains were carried on yeast nitrogen base (YNB) without amino acid (Difco Labs, Detroit) + 2% glucose agar plates with monthly transfers. Inocula were grown overnight at 30 C and 200 rpm in YNB without amino acid + 2% glycerol + phosphate buffer (pH 6.0).

<u>**Cell density determinations**</u>

Cell density was calculated by centrifuging whole fermentor broth for 10 minutes at a minimum of 4000 g and weighing the cell pellet to determine grams per liter of wet cells. A correlation factor of 1/4 is used to calculate dry cell concentrations.

<u>**Ethanol concentration determinations**</u>

Ethanol concentrations were determined by standard gas chromatography techniques.

<u>**Bovine lysozyme assays**</u>

Lysozyme concentrations were determined by a turbidimetric assay using lyophilized <u>Micrococcus lysodekticus</u> cells in a 100 mM sodium phosphate buffer, pH 5.0 - Shugar, <u>Biochem Biophys. Acta</u> <u>8</u>, 302 (1952). A linear decrease in absorbance (at 450 nm) was measured over 3 minutes at 30 C. The rate of decrease for fermentor samples was directly compared to previously purified recombinant bovine lysozyme c2 - See Dobson <u>et al.</u>, J. Biol. Chem. <u>259</u>, 11607 (1984) - to calculate concentrations.

<u>**Bovine lysozyme yield and productivity calculations**</u>

Concentrations (mg/L) of bovine lysozyme were calculated from the cell-free broth.

Total lysozyme (mg) was calculated based on the cell-containing whole broth from the fermentor. Volumetric productivity (mg/L-h) was calculated by dividing the total lysozyme produced by the liquid

volume in the fermentor and by the time of the induction phase. The time under repression and derepression is approximately 24-30 hours for these fermentations.

Yield of lysozyme per cell (mg/g) was calculated by plotting total lysozyme against total cells and using linear regression to determine the slope of the plot. The slope is taken as the yield of lysozyme per cell. The correlation coefficients for the slopes were greater than 0.95.

Fermentor start-up and general operation

The 2-liter fermentors (L.H. Fermentation, Hayward, CA; Biolafitte, LSL Biolafitte, Princeton, NJ) were autoclaved at a 700 ml volume containing 225 ml of 10X basal salts (42 ml/l 85% phosphoric acid, 1.8 g/l calcium sulphate-$2H_2O$, 28.6 g/l potassium sulfate, 23.4 g/l magnesium sulfate-$7H_2O$, 6.5 g/l potassium hydroxide) and 30 g glycerol. After sterilization, 3 ml of a YTM_4 trace salts solution (5.0 ml/l sulfuric acid, 65.0 g/l ferrous sulfate-$7H_2O$, 6.0 g/l copper sulfate-$5H_2O$, 20.0 g/l zinc sulfate-$7H_2O$, 3.0 g/l manganese sulfate-H_2O, 0.1 g/l biotin) was added and the pH adjusted to 5.0 with the addition of concentrated ammonium hydroxide; the pH was then controlled at 5.0 with the addition of a 20% ammonium hydroxide solution containing 0.1% Struktol J673 antifoam (Struktol Co., Stow, Ohio, USA) throughout the fermentation. Excessive foaming was sensed by a foam probe and controlled by addition of 2% Struktol J673 Antifoam. The fermentors were then inoculated with a 10-50 ml volume of inoculum. Upon exhaustion of the initial glycerol charge, a glycerol feed was started as described below. The dissolved oxygen of the fermentation was maintained above 20% of air saturation by increasing the air flow rate up to 3 liter/minute and agitation speed up to 1500 rpm during the fermentation.

Ten-liter fermentations (in a 14-liter Biolafitte fermentor) were started at: a 7.0 liter volume containing 1.9 liters 10X basal salts and 300 g glycerol for the Mut^- mixed fed-batch protocol, a 5.0 liter volume containing 2.4 liters of 10X basal salts and 360 g of glycerol for the Mut^+ methanol fed-batch protocol, or an 8 liter volume containing 3.2 liters of 10X basal salts and 480 g glycerol for the Mut^- methanol fed-batch protocol. After sterilization, 29 ml of a YTM_4 trace salts solution was added and the pH was adjusted and subsequently controlled at 5.0 with the addition of ammonia gas throughout the fermentation. Excessive foaming was controlled with the addition of 10% Struktol J673 Antifoam. The fermentor was inoculated with a volume of 200-500 ml. Upon exhaustion of the initial glycerol charge, a feed was started as outlined below. The dissolved oxygen was maintained above 20% by increasing the air flow rate up to 40 liter/minute, the agitation up to 1000 rpm and/or the pressure of the fermentor up to 1.5 bar during the fermentation.

<u>Mut^- (NL) mixed-feed fed batch fermentation</u>

After the glycerol batch phase was completed, a 50% (by weight) glycerol feed, containing 12 ml/l YTM_4 trace salts was started at 5.4 ml/h for the 2-liter fermentor or 54 ml/h for the 10-liter fermentor. After 6 hours of glycerol feeding, the glycerol feed was decreased to 3.6 ml/h (36 ml/h at 10-liters) and a methanol feed containing 12 ml/l YTM_4 trace salts was initiated at 1.1 ml/h for the 2-liter fermentor or 11 ml/h for the 10-liter fermentor. After 5 hours, the methanol feed was adjusted to give a residual methanol concentration of up to about 1%, preferably between 0.2 and 0.8%. The fermentation is carried out for 40-50 hours on the methanol and glycerol feed.

Mut$^-$ (2:1) mixed-feed fed batch fermentation

The 2:1 fermentation was carried out as the NL fermentation except that the methanol feed was not increased beyond 1.1 ml/hour, giving a 2:1 ratio of glycerol to methanol (by wt.) throughout the fermentation.

Mut$^-$ (4:1) mixed-feed fed batch fermentation

The 4:1 protocol was started in the same manner as the NL fermentation. During the simultaneous glycerol/methanol feeding, the 4:1 fermentation had a glycerol feed rate twice that used in the 2:1 protocol, i.e. 7.2 ml/hr for 1L and 72 ml/hr for 10L to give a 4:1 ratio of glycerol to methanol by weight throughout the fermentation.

Mut$^+$ (4:1) mixed-feed fed batch fermentation

This fermentation was conducted following the same protocol as for the Mut$^-$ strain in a 4:1 mixed-feed fermentation, for both a 1L and 10L fermentation.

Mut$^-$ methanol-fed-batch

After the glycerol batch phase was completed, an induced fed-batch phase was initiated by adding methanol to the fermentor to maintain a residual methanol concentration between 0.2 and 0.8%. For the 10L runs, the YTM_4 trace salts were not used. Instead, 40 ml of IM_1 trace salts (5 ml/l sulfuric acid, 4.8 g/l ferric chloride-$2H_2O$, 2.0 g/l zinc sulfate-H_2O, 0.02 g/l boric acid, 0.2 g/l sodium molybdate, 0.3 g/l manganese sulfate-H_2O, 0.08 g/l potassium iodide, 0.06 g/l copper sulfate-$5H_2O$) and 2 mg/l biotin were injected into the fermentor every two days.

Mut$^+$ methanol-fed-batch

After glycerol exhaustion, a 50% glycerol feed, containing 12 ml/l YTM_4 trace salts, was started

at 12 ml/h for the 2-liter or 200 ml/h for the 10-liter fermentor and run for a total of 7 hours. After 6 hours on the glycerol feed, the methanol feed, containing 12 ml/l YTM_4 trace salts, was started at 1.1 ml/h for the 2-liter and 11 ml/h for the 10-liter fermentor for 5 minutes. When a rise in dissolved oxygen was seen after the methanol feed was shut-off, the methanol feed was turned back on for another 5 minute interval. The latter process was repeated several times until an immediate response in the dissolved oxygen was observed to the methanol feed cessation; once this occurred, the methanol feed was increased by 20% per hour at 30 minute intervals. The methanol feed was increased until a feed rate of 7.6 ml/h for the 2-liter or 126 ml/h for the 10-liter fermentor was reached. The fermentation was then carried out for 40-60 hours for the 2-liter or 25-35 hours for the 10-liter fermentor.

<u>Alternative procedure for secreted product subject to degradation in broth</u>

A two liter LH fermentor containing 400 ml 10X basal salts, 80 g glycerol, and deionized water (to 1 liter) was sterilized. After sterilization and cooling, 3 ml YTM_4 solution was added and 20% NH_4OH used to bring pH to 3.6. The fermentor was inoculated with 60 ml of inoculum of Mut^- cells and the pH controller set at 5.0. During batch growth, the agitation speed was adjusted upward periodically to maintain a dissolved oxygen tension above 20% air saturation. After exhaustion of the initial glycerol charge, a 50% solution of glycerol containing 12 ml/L YTM_4 was pumped into the fermentor at the rate of 20 ml/h. Four and one-half hours later, the glycerol feed rate was decreased to 10 ml/hr and a feed of methanol containing 12 ml/L YTM^4 was started at 1.0 ml/h. Three hours later the methanol feed rate doubled. After

ninety minutes at 2 ml/h, the methanol feed rate was adjusted to 3.8 ml/h and maintained constant until harvest at 13.5 hours after the methanol feed was first initiated.

<u>Mut⁻ mixed-feed continuous culture</u>

A 14L Biolafitte fermentor containing 7.6 kg sterile medium comprised of 3.2L 10X basal salts, 800 g glycerol, 32 ml YTM_4 trace salts was inoculated with 500 ml of an overnight culture of strain A37 in YNB + 2% glycerol + phosphate buffer. The fermentation was run at 30°C and controlled at pH5 by addition of NH_3. Excess foaming was controlled by addition of Strucktol J-673 Antifoam.

When the initial batch charge of glycerol was exhausted, a 50% glycerol feed containing 12ml/L YTM_4 and 12ml/L IM_1 trace salts was initiated at 200ml/h. After 6 hours, the glycerol feed was reduced to 122ml/h and a methanol feed was initiated at 19ml/h. Eight hours after the initiation of the methanol feed, a 4X basal salts feed was initiated and the fermentor volume was controlled at 8.4L by an automatic weight controller. Four hours later all feed rates were adjusted downward in response to ethanol accumulation. The adjusted feed rates were 60ml/h, 14ml/h and 32ml/h for salts, methanol, and glycerol respectively.

The fermentation was run 20 hours at the above flow rates and then the rates were increased to 156ml/h, 41ml/h and 88 ml/h while the volume was decreased to 7.4L. The following day the feeds were decreased to 119ml/h, 30ml/h and 65ml/h and left constant for two days. A final feed adjustment left the flow rates at 80ml/h, 30ml/h and 36ml/h for salts, methanol and glycerol for two additional days.

Following the initial downward adjustment of the feed rates 4 hours into the continuous phase, the lysozyme concentration in the fermentor continuously

increased, rising from 110mg/L to 525mg/L at the final sampling, resulting in a volumetric productivity of 10mg/L-h in the continuous process.

Mut^+ mixed-feed continuous culture

In a 14L fermentation run with a Mut^+ strain it was also found that manipulation of the feed composition could enhance the lysozyme productivity. Decreasing the glycerol:methanol ratio from 4:1 to 2:1 increased the volumetric productivity from 5mg/Lh to 9mg/L-h.

It should be noted that the volumetric productivity is based on lysozyme values which have been corrected for cell density. The productivity is based on the amount of lysozyme per liter of fermentor broth rather than per liter of cell-free broth. At the cell densities encountered in this work, the concentration in the cell-free broth can be 25-40% higher than the whole broth values.

A comparison of the various fermentation modes employed with Mut^- and Mut^+ recombinant cultures is presented in Table 1 for fermentations run at the 1L scale.

TABLE 1

1 LITER FERMENTATION - LYSOZYME

	CH_3OH FED MUT^-	MIXED-FED MUT^- 4:1	MIXED-FED MUT^- 2:1	MIXED-FED MUT^- NL	CH_3OH FED MUT^+	MIXED-FED MUT^+ (4:1)
MAXIMUM LYSOZYME CONCENTRATION (mg/l)	250	180	290	375	450	130
TIME INDUCED (hours)	175	39	43	45	39	49
CELL DENSITY (dry g/l)	60	82	85	103	84	78
VOLUMETRIC PRODUCTIVITY (mg/l-h)	1.2	3.4	4.8	5.6	7.7	3.0
LYSOZYME YIELD PER CELL (mg/g)	5.2	2.3	3.7	4.0	5.6	2.4
MAXIMUM ETHANOL CONCENTRATION (mg/l)	10	210	100	100	30	N.D.

Several significant observations from Table 1 are the following:

1. The volumetric productivities of the Mut$^-$ strains grown in the invented mixed-feed mode exceed the volumetric productivity of the Mut$^-$ strain grown in the methanol only feed mode by 3-6-fold.

2. The maximum concentration of the bovine lysozyme secreted by the Mut$^-$ cells grown in the mixed-feed modes of fermentation approximate (180 mg/L) and exceed (290 and 375 mg/L) that secreted by the same cells grown in the methanol only feed mode (250 mg/L). Significantly, these mixed-feed concentrations are reached in approximately one-quarter of the time.

3. The mixed-feed fermentation modes allow the Mut$^-$ cells to attain a higher cell density than that attained by the same cells grown in the methanol only feed mode. Again, the higher cell densities are reached in one-quarter the time.

4. The non-limiting (NL), mixed-fed fermentation mode was preferred for the Mut$^-$ strains.

Figure 1 shows the lysozyme production in a 2-liter fermentor under the four different protocols: methanol feed alone or a mixed-feed of glycerol and methanol at a 4:1, 2:1 (glycerol:methanol by weight), or a limited glycerol, non-limited methanol (NL) feed. The NL mixed-feed fermentation yielded 375 mg/L in 48 hours, giving a 6-fold increase in volumetric productivity over the original methanol only Mut$^-$ fermentation.

Figure 2 shows a comparison of the methanol only-fed Mut$^-$ and the mixed-fed NL Mut$^-$ fermentations to the methanol only-fed Mut$^+$ fermentation. The data in this Figure show that the productivity of slower-growing Mut$^-$ cells can be made equivalent to the faster growing Mut$^+$ cells by growing the Mut$^-$ cells in the invented mixed-feed mode of fermentation. Because the Mut$^-$ cells are easier to maintain in a fermentor than

the Mut^+ cells, primarily due to the latter's sensitivity to ethanol build up, the ability to grow the Mut^- strains in such a way as to achieve comparable productivity to the Mut^+ is an advantage.

Figure 3A shows a comparison of the 10 liter and 1 liter results. The higher product levels and productivity were expected for the 10 liter fermentation since a higher starting glycerol concentration was used (7% vs. 4%). This 10 liter fermentation reached 325 mg/l in 125 hours (Table 2) giving a volumetric productivity of 2.1 mg/l-h for the induction phase. The yield of 5.2 mg/g cell was the same as that obtained in the 1 liter fermentations.

Lysozyme concentration in a typical 10 liter run is shown in Figure 3B, the rate of lysozyme production slows after 20 hours. The volumetric productivity is higher (5.8 mg/l-h) than the methanol-fed fermentations.

TABLE 2

SCALE-UP TO 10L OF BOVINE LYSOZYME FERMENTATIONS

	CH_3OH FED MUT^-	MIXED-FED (NL) MUT^-
MAXIMUM LYSOZYME CONCENTRATION (mg/l)	325	220
TIME INDUCED (hours)	120	30
CELL DENSITY (dry g/l)	75	80
VOLUMETRIC PRODUCTIVITY (mg/l-h)	2.1	5.8
LYSOZYME YIELD PER CELL (mg/g)	5.2	2.8
MAXIMUM ETHANOL CONCENTRATION (mg/l)	10	220

Volumetric productivity increased dramatically with the mixed-fed (NL) versus methanol-fed fermentations.

2. <u>Human Lysozyme</u>

A plasmid, designated pHLZ103, was constructed by inserting a DNA with the following sequence:

5'-AATTCATGAAGGCTCTCATTGTTCTGGGGCTTGTCCTCCTTTCTGTTACG
GTCCAGGGCAAGGTCTTTGAAAGGTGTGAGTTGGCCAGAACTCTGAAAAGATT
GGGAATGGATGGCTACAGGGGAATCAGCCTAGCAAACTGGATGTGTTTGGCCA
AATGGGAGAGTGGTTACAACACACGAGCTACAAACTACAATGCTGGAGACAGA
AGCACTGATTATGGGATATTTCAGATCAATAGCCGCTACTGGTGTAATGATGG
CAAAACCCCAGGAGCAGTTAATGCCTGTCATTTATCCTGCAGTGCTTTGCTGC
AAGATAACATCGCTGATGCTGTAGCTTGTGCAAAGAGGGTTGTCCGTGATCCA
CAAGGCATTAGAGCATGGGTGGCATGGAGAAATCGTTGTCAAAACAGAGATGT
CCGTCAGTATGTTCAAGGTTGTGGAGTGTAAG

into the <u>Eco</u>RI site of pAO804. The DNA with the above sequence, excluding the first 5 bases (corresponding to an <u>Eco</u>RI site) at the 5'-end and the final base (corresponding to an <u>Eco</u>RI site) at the 3'-end, encodes a pre-lysozyme which has a signal peptide that has the same amino acid sequence as the signal peptide of the pre-lysozyme of human placental origin (Castanon <u>et al.</u>, Gene <u>66</u>, 223-234 (1988)) and the signal peptide of the pre-lysozyme of human histiocytic lymphoma cell line U-937 (Sundstrom and Nilsson, <u>Intl. J. Cancer</u> <u>117</u>, 565-577 (1976)) and which has a mature lysozyme peptide that has the same amino acid sequence as human milk lysozyme (Jolles <u>et al.</u>, <u>Mol. and Cell. Biochem.</u> <u>63</u>, 165 (1984)), lysozyme of human placental origin (Castanon <u>et al.</u>, <u>supra</u>), and lysozyme of human histiocytic lymphoma cell line U-937 (Sundstrom and Nilsson, <u>supra</u>).

Plasmid pHLZ103 was used, as described above in connection with bovine lysozyme c2, to prepare, from <u>P. pastoris</u> strain GS115, Mut$^-$, Mut$^+$ (single copy), and

Mut+ (multicopy) strains, in which pre-human lysozyme is made and from which mature human lysozyme is secreted into the medium.

The human lysozyme-secreting strains were cultured by the various methods described above in connection with bovine lysozyme-secreting strains and, thereby, human lysozyme was made and recovered from the culture media. The advantages noted above for mixed-feed fermentation protocols with bovine lysozyme-producing strains of methylotrophic yeast are observed as well with the human lysozyme-producing strains.

3. <u>Epidermal Growth Factor</u>

<u>Plasmids</u>

A DNA was constructed with the following sequence:

5'-AATTCATGAGAATTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCC
TCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAAT
TCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTG
CTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACT
ACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTTTCTTTGGATAAAAGAAA
TTCCGATAGCGAGTGTCCTCTGAGTCACGATGGTTACTGTCTACATGACGGCG
TCTGTATGTATATTGAGGCTCTAGACAAGTACGCGTGTAATTGCGTTGTTGGC
TACATCGGTGAGCGTTGTCAGTATCGTGATCTGAAATGGTGGGAACTTCGTTA
AAGCTGG

The first five bases at the 5'-end of the sequence correspond to an <u>Eco</u>RI site. The G at the 3'-end of the sequence corresponds also to an <u>Eco</u>RI site. The 5'-AGCTG immediately 5' of the G at the 3'-end of the sequence is an artifact of the procedure used to construct the DNA. The remaining 417 bases of the sequence, beginning with 5'-ATG near the 5'-end and ending with the translation stop signal encoding triplet, 5'-TAA, near the 3'-end, code for the N-terminal 85 amino acids of prepro-alpha mating factor of <u>Saccharomyces</u> <u>cerevisiae</u> (see U. S. Patent

No. 4,546,082) and the 53 amino acids of mature, 53-amino acid, human epidermal growth factor (i.e., EGF(1-53)). The DNA is inserted into the EcoRI site of plasmid pAO804, and, as described above in connection with bovine and human lysozymes, the resulting plasmid, designated pAO817', is used with P. pastoris strain GS115 to prepare Mut$^-$, Mut$^+$ (single copy), and Mut$^+$ (multicopy) strains, from which, on culturing in the various modes described above, mature, 52-amino acid, human epidermal growth factor (i.e., EGF(1-52)) and mature, 48-amino acid, human epidermal growth factor (i.e., EGF(1-48)) are secreted into the culture medium. The secretion is mediated by the N-terminal 85 amino acids (from S. cerevisiae prepro-alpha mating factor) of the polypeptide encoded by the DNA with the above sequence. It appears that the carboxy-terminal amino acid of that polypeptide is rapidly cleaved by proteolysis in the P. pastoris strains or in the medium, such that mature EGF(1-53) is not found to be a significant fraction of the EGF recovered. Although, as noted, mature EGF(1-52) is isolated from the culture media, it is slowly converted by proteolysis in the media to the stable, mature EGF(1-48). (EGF(1-52) and EGF(1-48) have the same biological activities as EGF(1-53) and, like EGF(1-53), are therapeutically useful.)

As with making the bovine and human lysozymes in methylotrophic yeast, the advantages of using mixed-feed protocols were observed as well with all of the various Mut phenotypes in making EGF(1-52) and EGF(1-48).

EGF(1-52) and EGF(1-48) were also made by culturing strains of P. pastoris GS115 that had been transformed, by the spheroplast method (Cregg et al., Mol. Cell. Biol. 5, 3376 (1985)), with BglII-cut plasmid pAO817 (to yield Mut$^-$ strains with the genomic AOX1 gene disrupted by integration of EGF-encoding DNA)

or, also by the spheroplast method, with intact pAO817 (to yield, by addition into a region of homology in the P. pastoris genome, Mut^+ strains). In plasmid pAO817, there are two, identical expression cassettes joined to each other in head-to-tail arrangement. Each of the cassettes comprises the P. pastoris AOX1 promoter joined operably for transcription to the approximately 430-bp, prepro-alpha mating factor (1-85), mature EGF(1-53)-encoding DNA with the sequence listed above and, downstream of that DNA, the polyadenylation signal-encoding and polyadenylation site-encoding DNA segments and transcription terminator of the P. pastoris AOX1 gene.

The ≈430 bp EcoRI-site-terminated, prepro-alpha mating factor, mature EGF-encoding DNA segment, of sequence indicated above, was isolated on a 1.5% agarose gel. 15 μg of plasmid pAO815 (the construction of which is described below) was digested with EcoRI and ligated to the ≈430 bp fragment in a standard ligation reaction. To determine which transformants had a plasmid with the correct orientation of the ≈430 bp fragment (for transcription from the AOX1 promoter to make an mRNA encoding the prepro-alpha mating factor(1-85)-EGF(1-53) fusion), plasmid DNA was digested with PstI. The correct construct yielded an ≈1740 bp fragment. The correct plasmid was named pAO816.

The complete, AOX1-promoter-driven expression cassette for the prepro-alpha mating factor(1-85)-EGF(1-53) fusion was removed from pAO816 by digesting 15 μg of pAO816 with BglII and BamHI and isolating the ≈1670 bp fragment on a gel. The gel-purified fragment was then ligated to BamHI-cut pAO816. The ligation mix was used to transform E. coli MC1061 and amp^R colonies were selected. Colonies having plasmid with two head-to-tail expression cassettes were identified by

digestion with PstI, which gave fragments of 1827, 1497 and 9547 bp. This plasmid was named pAO817.

Plasmid pAO815 was constructed by mutagenizing plasmid pAO807 (described below) to change the ClaI site downstream of and near the AOX1 transcription terminator (see description above of construction of pAO804) in pAO807 to a BamHI site. The oligonucleotide used for mutagenizing pAO807 had the following sequence: 5'-GACGTTCGTTTGTGCGGATCCAATGCGGTA GTTTAT. The mutagenized plasmid was called pAO807-Bam. Plasmid pAO804 was digested with BglII and 25 ng of the 2400 bp fragment was ligated to 250 ng of the 5400 bp BglII fragment from BglII-digested pAO807-Bam. The correct construct was verified by digesting with PstI/ BamHI to identify bands of 6100 and 2100 bp. The correct construct was called pAO815.

Plasmid pAO807 was constructed as follows:

An ≈458 bp, RsaI-DraI DNA fragment (called "f1-ori fragment") containing the origin of replication (ori) or bacteriophage f1 was obtained from the phage by a standard procedure.

pBR322 (2 μg) was partially digested with 2 units of DraI. The ligation mixture was used to transform *E. coli* strain JM103. Amp^R transformants were pooled and superinfected with helper phage R408. Single stranded phage were isolated from the media and used to reinfect JM103. Amp^R transformants contained the plasmid pBRf1-ori, which contains f1-ori cloned into the DraI sites (nucleotide positions 3232 and 3251) of pBR322.

pBRf1-ori (10 μg) was digested with PstI and NdeI and the ≈0.8 kbp fragment containing the f1-ori was isolated by electrophoresis on a 1.2% agarose gel. About 100 ng of this DNA was mixed with 100 ng of pAO804 that had been digested with PstI and NdeI and phosphatase-treated. This mixture was ligated and then used to transform *E. coli* JM103 to obtain pAO807.

Strains

Mut$^-$ strains

20 μg of the expression vector pAO817 were digested with BglII, which released the tandem expression cassettes. The linear DNA fragment obtained by digestion (5 μg) was transformed into P. pastoris strain GS115 (ATCC 20864) by the spheroplast method [Cregg et al., Mol. Cell. Biol. 5, 3376 (1985)]. His$^+$ cells were selected and the methanol utilization phenotype (Mut) of the cells was determined.

Approximately 15% of the cells were His$^+$Mut$^-$, indicating that the expression vector integrated correctly at the AOX1 locus and disrupted the AOX1 gene. Southern analysis of the EcoRI digest of the transformants, using the plasmid pAO803 as probe, confirmed the disruption of the AOX1 gene and showed the number of expression units integrated. Strains selected for further study were named as indicated in Table 3.

TABLE 3

Strain Name	Phenotype	Site of Integration	Copy Number
G-EGF817S10	Mut$^-$His$^+$	AOX1	One
G-EGF817S7	Mut$^-$His$^+$	AOX1	One
G-EGF817S9	Mut$^-$His$^+$	AOX1	Multiple

In Table 3, "copy number" refers to the number of BglII fragments integrated. Each BglII fragment has two expression cassettes for the prepro-alpha mating factor(1-85) - EGF(1-53) fusion.

Mut$^+$ strains

P. pastoris strain GS115 (ATCC 20864) was transformed with 5 μg of uncut vector pAO817 using the spheroplast method of transformation. In this type of transformation the plasmid will integrate by addition into the P. pastoris genome at a site of homology with

a segment on the plasmid. The transformants were screened for His^+Mut^+ phenotype, and several were picked for Southern analysis. An EcoRI digest was probed with plasmid pYM4 [pYM4 was obtained by digestion pYM30 (NRRL B-15890) with ClaI and religating the ends] and the hybridization pattern revealed two of the six had appropriate integrations. Strains selected for further work are listed in Table 4.

TABLE 4

Strain Name	Phenotype	Site of Integration	Copy Number
G+EGF817S1	Mut^+His^+	HIS4	One
G+EGF187S6	Mut^+His^+	HIS4	One

Fermentation of EGF-Producing Strains

a. Fermentor start-up and general operation

The 2-liter fermentors (L.H. Fermentation, Hayward, CA; Biolafitte, LSL Biolafitte, Princeton, NJ) were autoclaved at a 700 ml volume containing 225 ml of 10X basal salts (52 ml/l 85% phosphoric acid, 1.8 g/l Calcium Sulphate-$2H_{20}$), 28.6 g/l Potassium Sulfate, 23.4 g/l Magnesium Sulfate-7H2O), 6.5 g/l Potassium Hydroxide) and 30 g glycerol. After sterilization, 3 ml of a YTM_4 trace salts solution was added and the pH adjusted to 5.0 with the addition of concentrated ammonium hydroxide; the pH was then controlled at 5.0 with the addition of a 20% ammonium hydroxide solution containing 0.1% Struktol J673 antifoam throughout the fermentation. Excessive foaming was controlled throughout the fermentation by addition of Struktol J693 antifoam when foam contacted a foam sensor in the fermentor. The fermentors were then inoculated with a 10-50 ml volume of inoculum (overnight shake flask culture in phosphate-buffered 0.65% Yeast Nitrogen Base, pH6, containing 2% glycerol). Upon exhaustion of the initial glycerol charge, a glycerol feed was started as described below. The dissolved oxygen of

the fermentation was maintained above 20% of air saturation by increasing the air flow rate up to 3 liter/minute and agitation speed up to 1500 rpm during the fermentation.

Ten-liter fermentations (in a 15-liter Biolafitte fermentor) were started in a 7.0 liter volume containing 4 liters of 10X basal salts and 520 g of glycerol for the Mut^+ methanol fed-batch protocol. After sterilization, 30 ml each of YTM_4 and IM_1 trace salts solutions were added and the pH was adjusted and subsequently controlled at 5.0 with the addition of ammonia gas throughout the fermentation. Excessive foaming was controlled with the addition of 5% Struktol J673 antifoam. The fermentor was inoculated with a volume of 200-500 ml. Upon exhaustion of the initial glycerol charge, a feed was started as outlined below. The dissolved oxygen was maintained above 20% by increasing the air flow rate up to 40 liter/minute, the agitation up to 1000 rpm and/or the pressure of the fermentor up to 1.5 bar during the fermentation.

b. Growth of Mut^- strains in one-liter fermentors

(1) Mut^- (NL) mixed-feed fed batch fermentation

After the glycerol batch phase was completed, a 50% (by weight) glycerol feed, containing 12 ml/l YTM_4 trace salts was started at 5.4 ml/h for the 2-liter fermentor. After 6 hours of glycerol feeding, the glycerol feed was decreased to 3.6 ml/h (36 ml/h at 10-liters) and a methanol feed containing 12 ml/l YTM_4 trace salts was initiated at 1.1 ml/h for the 2-liter fermentor. After 5 hours, the methanol feed was adjusted to give a residual methanol concentration of up to about 1%, preferably between 0.2 and 0.8%. The fermentation was sampled periodically and harvested 36-50 hours after the methanol feed was initiated.

(2) Mut^- methanol-fed-batch

After the glycerol batch phase was completed, an induced fed-batch phase was initiated by adding methanol to the fermentor to maintain a residual methanol concentration between 0.2 and 0.8%. The fermentor was sampled periodically and harvested after 167 hr growth on methanol.

(3) Alternative procedure for production of EGF(1-52)

A two liter LH fermentor containing 400 ml 10X basal salts, 80 g glycerol, and deionized water (to 1 liter) was sterilized. After sterilization and cooling, 3 ml YTM_4 solution was added and 20% NH_4OH used to bring pH to 3.6. The fermentor was inoculated with 60 ml of inoculum of Mut^- cells and the pH controller set at 5.0. During batch growth, the agitation speed was adjusted upward periodically to maintain a dissolved oxygen tension above 20% air saturation. After exhaustion of the initial glycerol charge, a 50% solution of glycerol containing 12 ml/l YTM_4 was pumped into the fermentor at the rate of 20 ml/h. Four and one-half hours later, the glycerol feed rate was decreased to 10 ml/hr and a feed of methanol containing 12 ml/l YTM_4 was started at 1.0 ml/h. Three hours later the methanol feed rate was doubled. After ninety minutes at 2 ml/h, the methanol freed rate was adjusted to 3.8 ml/h and maintained constant until harvest at 13.5 hours after the methanol feed was first initiated.

c. Growth of Mut^+ strains in two-liter fermentors - Mut^+ methanol-fed-batch

After glycerol exhaustion, a 50% glycerol feed, containing 12 ml/l YTM_4 trace salts, was started at 12 ml/h for the 2-liter fermentor and run for a total of 7 hours. After 6 hours on the glycerol feed,

the methanol feed, containing 12 ml/l YTM_4 trace salts, was started at 1.1 ml/h for 5 minutes. When a rise in dissolved oxygen was seen after the methanol feed was shut-off, the methanol feed was turned back on for another 5 minute interval. The latter process was repeated several times until an immediate response in the dissolved oxygen was observed to the methanol feed cessation; once this occurred, the methanol feed was increased by 20% per hour at 30 minute intervals. The methanol feed was increased until a feed rate of 7.6 ml/h was reached. The fermentation was then carried our for 40-60 hours.

d. Results of fermentations

Under the methanol fed batch protocol, cell growth for both strains G-EGF817S9 and G-EGF817S10 was similar, yielding about 300 g/l wet cells after 167 h. However, the multicopy strain G-EGF817S9 produced 400 mg/l of EGF, twice as much as the other strain with only two copies of the EGF expression cassette. The maximum concentration of EGF was reached after 120 hours growth on methanol.

Under the mixed-feed protocol, both strains again grew up to more than 300 g/l, and the 400 mg/l of EGF produced by the multicopy strain was again higher than that produced by the double copy strain. The strains grew significantly more rapidly in the mixed fed mode.

With the multicopy strain, reduced time on methanol was require to produce EGF using mixed feed compared to using methanol alone, 35 hr vs. 120 hr respectively. The initial batch growth on glycerol to build up cell mass adds another 24 h to the overall process time. The EGF productivities for the methanol and mixed feed modes are 3 $mgl^{-1}h^{-1}$ and 7 $mgl^{-1}h^{-1}$, respectively.

A Mut$^+$ strain carrying two copies of the EGF gene, G+EGF817S1, produced hEGF at concentrations similar to those seen in a Mut$^-$ strain carrying two copies of the hEGF gene.

e. Stability of secreted EGF in fermentation broth of Pichia pastoris

HPLC analysis of EGF in the broth during the time course of the fermentation runs revealed that the 1-48 peptide was much more stable than the longer forms. The longer forms could be seen early after induction during a run. After 24h growth on methanol, the 1-48 peptide would accumulate, apparently as a degradation product of the other forms. The 1-48 peptide was very stable under fermentation conditions, persisting and accumulating for up to six days in the longer fermentation protocols. This unexpected high stability makes production and purification of this form of hEGF much simpler than that of the longer forms.

f. Biological activity of EGF (1-48)

The 1-48 EGF peptide was tested for biological activity both in *in vitro* cell mitogenic assays and *in vivo* in stimulation of gastric ulcer healing. The peptide was observed to have high biological activity in both types of tests.

4. **Human Superoxide Dismutase**

Plasmid pSOD104

Plasmid pSOD104 was made by inserting, into the EcoRI site of pAO804, the approximately 470 bp DNA with EcoRI sites on both ends bracketing a segment with the sequence that, but for the two exceptions noted below, is identical to that of the 465 base pairs encoding 154 amino acids and the translational stop

codon shown in Figure 1 of Hallewell et al., Nucl. Acids Res. 13, 2017 - 2034 (1985). The two exceptions are that, in the insert in pSOD104, the last base of the stop codon-encoding triplet is G rather than the A in Hallewell et. al. and, in the insert in pSOD104, the last base of the triplet encoding Thr-54 is a G rather than the A in Hallewell et al. In pSOD104, the insert into the EcoRI site of pAO804 encodes the subunit of human erythrocyte Cu/Zn superoxide dismutase (referred to herein as "human superoxide dismutase" or "human SOD").

Strains of P. pastoris made by transformation with pSOD104 or fragments thereof make human superoxide dismutase intracellularly. The human SOD obtained from these cells has the activity of authentic human SOD and is useful in the same applications in which SODs, and particularly human SOD, are used, including therapeutic applications.

Strains

Plasmid pSOD104 was used to develop both Mut^+ and Mut^- strains of P. pastoris. The host strain was the histidine-requiring auxotroph GS115 (ATCC No. 20864). Transformation was carried out by the spheroplast method described by Cregg et al., Mol. Cell. Biol. 5, 3376 (1985). To develop Mut^+ strains, undigested pSOD104 was transformed into GS115 and His^+ cells were selected. Nine His^+ prototrophs were examined by Southern hybridization analyses. The chromosomal DNAs were digested with EcoRI and probed with pAO803, containing AOX1 5' and 3' regions, or pYM4 containing the Pichia HIS4 gene. A BglII digestion was also performed and probed with an oligonucleotide of sequence 5'-AACTCATGAACATGGAATCCATGCAGGCCT, which is homologous to a segment of the hSOD gene. The results of the Southern identified three classes of Mut^+ transformants which are summarized in Table 5 below:

TABLE 5

Strain Name	Copy Number	Site of Integration
G+SOD104C1,4,5,7,8	one	AOX1
G+SOD104C2,9,10	one	HIS4
G+SOD104C3	two	HIS4 and AOX1

To develop Mut^- strains, a BglII digest of plasmid pSOD104 was transformed into GS115 cells, and His^+ cells were selected. The His^+ prototrophs were then screened for their Mut phenotype.

Approximately 12% of the transformants were slow growers, indicative of the disruption of the AOX1 locus. Twenty two of these were compared with the control strain, G-PAO804, for growth rates on methanol. Strain G-PAO804 was developed by disrupting the AOX1 locus with a BglII digest of plasmid pAO804. It displays the expected Mut^- phenotype but it does not express a recombinant gene product. All hSOD Mut^- transformants appeared to grow at approximately the same rate as G-PAO804. A slower growth rate can be indicative of toxicity to the cells from the heterologous gene product.

Nine of the His^+ Mut^- cells were analyzed by Southern blots, as described hereinabove. All nine strains were shown to have integrated one copy of the expression cassette at the AOX1 locus, thus disrupting the gene. These strains were named G-SOD104C1 through G-SOD104C9.

Fermentation

Based on shake-flask expression results, two of the Mut^+ strains, G+SOD104C10 (one copy) and G+SOD104C3 (two copies) and one of the Mut^- strains, G-SOD104C5, were evaluated in 1L fermentors for production of hSOD. The Mut^+ cells were grown in a methanol-limited fed-batch mode, and the Mut^- cells were

grown in both a methanol-excess fed-batch and a mixed-feed fed-batch mode as follows:

1. Methanol-fed-batch, limited MeOH, Mut^+ phenotypes

The fermentor was autoclaved with 700 ml basal salts medium (final basal salts concentration of 3.3X) (10X basal salts: Phosphoric Acid(85%) 42.0 ml/L, Calcium Sulfate$\cdot 2H_2O$ 1.8 g/L, Potassium Sulfate 28.6 g/L, Magnesium Sulfate$\cdot 7H_2O$ 23.4 g/L, Potassium Hydroxide 6.5 g/L) and 4% glycerol. After sterilization, 3 ml each of YTM_4 and IM_1 were added and the pH was brought to 5 with concentrated NH4OH. Afterward, pH5 was maintained by addition of dilute (1:4) NH_4OH containing 0.1% Struktol J673 antifoam. Inocula were prepared from selective plates and grown overnight at 30°C in phosphate-buffered 0.67% yeast nitrogen base (pH 5) containing 2% glycerol. The fermentor was inoculated with cultured cells and the batch growth regime lasted 18 to 24 hours. At the point of substrate exhaustion, a 50% glycerol feed (containing 12 ml/L each of YTM_4 and IM_1) was initiated at 12 ml/h for 7 h. At the point of substrate exhaustion, usually after six hours of glycerol feeding, an MeOH feed (containing 12 ml/L each of YTM_4 and IM_1) was initiated at 1.5 ml/hr and an additional 3 ml of YTM_4 and IM_1 were added to the fermentor. Adjustments in increments of 10% every 30 min were made over the course of the next 10 hours until a final feed rate of 7.5 ml/h was attained. The vessel was harvested 48-80 hours following MeOH induction.

2. Limited MeOH, continuous culture, Mut+ phenotype

The fermentor was prepared as described for the limited MeOH-fed-batch protocol hereinabove. Upon glycerol exhaustion, a 5% MeOH feed (containing 4X Basal Salts, 12 ml YTM_4 and IM_1 per liter of MeOH) was initiated at 10 ml/h. The 5% MeOH feed was increased

to 60 ml/h over the next six hours. Once the reactor liquid volume reached 1 liter (approximately 30 hours), a harvest stream was initiated at a rate equal to the feed rate to maintain a constant volume of 1 liter in the fermentor.

At 143 hours on MeOH, 1 ml of a 1% copper and zinc solution was added to the reactor. After 295 hours on MeOH, the feed and effluent rates were decreased to 30 ml/h. Continuous culture was switched to fed-batch mode after 431 hours by switching from the 5% MeOH feed to a 100% MeOH feed (plus 12 ml/L YTM_4 and IM_1) and terminating the effluent stream. The fed-batch mode was run as described above, for a period of 72 hours for a total of 503 hours of MeOH for the entire run.

3. Methanol-fed-batch, excess MeOH, Mut- phenotypes

The fermentor was prepared and inoculated as described above for a Mut+ methanol-fed-batch run. At the point of substrate exhaustion a methanol feed (containing 12 ml/L each of YTM_4 and IM_1) was initiated at 0.5 ml/h and an additional 3 ml each of YTM_4 and IM_1 were added to the fermentor. The feed rate was increased over the course of the fermentation to maintain a residual MeOH concentration of approximately 4 g/l. The vessel was harvested from 6 to 10 days following MeOH induction.

4. Mixed-feed fed-batch, Mut- phenotype

The fermentor was prepared and inoculated as described above for Mut+ methanol-fed-batch run. At the point of substrate exhaustion a 50% glycerol feed (containing 12 ml/L each of YTM_4 and IM_1) was initiated at 5.4 ml/h for 6 hours. Then, the glycerol feed rate was reduced to 3.6 ml/h, 3 ml each of YTM_4 and IM_1 were added, and a MeOH feed (containing 12 ml/L each of YTM_4 and IM_1) was started. The initial rate of MeOH addition was about 1 ml/h (MeOH:glycerol feed ratio of 0.7:1).

Adjustments in increments of 10% every 30 minutes were made over the course of the next 10 hours until a final feed rate of 4.9 ml/h (MeOH:glycerol feed ratio of 2:1) was attained. The MeOH feed rate was adjusted so that the residual MeOH concentration did not exceed 4 g/l. The vessel was harvested 80 hours following MeOH induction.

Results

The Mut+ strains (double and single copy, respectively) attained high cell density (340-350 g WW/L) in only 54 hours, employing the methanol-fed-batch protocol (Fermentation Mode 1). ("WW" means "wet weight".) The methanol-fed-batch protocol routinely used for Mut- strains (Fermentation Mode 3) is a much longer fermentation, typically lasting from 6 to 10 days. For example, in one such run, 150 hours were required to attain 280 g WW/L cell density. Using a mixed-feed strategy (Fermentation Mode 3), it was demonstrated that a Mut- strain could be quickly grown to a density equivalent to the Mut+ strains; 380 g WW/L (95 g dry weight/L) cell density was achieved in 80 hours.

The time-course of hSOD expression in Modes 1, 3 and 4 showed that in these runs the highest hSOD levels (3500 kU/L, or 0.92 g/L based on a specific activity of 3.8 U/μg) were produced by a double-copy, Mut+ strain. This strain also demonstrated one of the highest specific productivities, 240 U/g WW-h, as set forth in Table 6.

Among the single-copy strains, expression level and specific productivity were highest in the Mut+ strains, followed by the Mut- strains grown on a mixed substrate. The Mut- cells grown in a methanol-limited fed-batch mode were less productive than the others.

As indicated by similar yields and specific productivities (Table 6), the earlier runs in Mode 1 were reproduced successfully in the later runs in that mode. The latter two Runs in that mode were induced on methanol for 80 hours. Since the yield of hSOD is proportional to the amount of cell mass produced under inducing conditions, higher expression levels, 5100 kU/L and 1400 kU/L, respectively, were obtained in the longer runs. Based on our determination of hSOD specific activity, the best level of expression was realized in a later, mode 1 run. 1.3 g hSOD was produced per liter of fermentor volume in that run.

TABLE 6

hSOD-EXPRESSING PICHIA STRAINS

RUN	STRAIN	PHENO-TYPE	COPY #	MAX SOD kU/L	MAX SOD g/L	HRS	MAX Wet Wt. g/L	SPECIFIC PRODUCTIVITY U/g/h	VOLUMETRIC PRODUCTIVITY kU/L/h
Mode 1									
441	G+SOD104C3	Mut^+	2	3500	0.92	54	340	240	62
442	G+SOD104C10	Mut^+	1	1100	0.29	54	359	72	18
459	G+SOD104C10	Mut^+	1	1400	0.37	79	421	58	16
460	G+SOD104C3	Mut^+	2	5100	1.34	80	411	220	66
Mode 2									
476[1]	G+SOD104C3	Mut^+	2	640	0.17	431	80	330	38
476[2]	G+SOD104C3	Mut^+	2	4600	1.21	503	350	204	55
Mode 3									
445	G-SOD104C5	Mut^-	1	700	0.18	143	277	26	5.9
Mode 4									
446	G-SOD104C5	Mut^-	1	920	0.24	71	382	50	13

1 Run 476 was conducted first in a continuous culture mode, followed by a fed-batch mode. In the continuous culture mode hSOD levels were maintained for 431 hr and were insensitive to the addition of copper and zinc to the reactor.

2 Fed-batch mode of Run 476.

As hereinabove described, Run 476 was conducted first in a continuous culture mode, followed by a fed-batch mode. In the continuous culture, hSOD levels were maintained for 431 hours and were insensitive to the addition of copper and zinc to the reactor. As illustrated in Table 6, the double-copy strain G+SOD104C3 grown following this fermentation protocol gave the best specific productivity (330 U/g/h).

Comparison of the results in Table 6 for runs 445 and 446 demonstrates the advantages of mixed-feed fermentation..

The above-discussed hSOD expression levels are based on data from activity assays described below. Since activity assays can only measure functional molecules, denatured or inactive enzymes would escape detection. Therefore, the indicated expression levels are lower limits; the true levels are equal to or greater than these values.

Human SOD Activity Assay

The nitrite assay [Y. Oyanagui, Anal. Biochem. 142, 290 (1984)] was used to measure hSOD activity. This assay is based on the oxidation of hydroxylamine by hSOD to nitrite that is then detected with an indicator dye.

The nitrite assay has proved to be particularly successful for measuring hSOD concentrations in Pichia lysates. Table 7 shows that several samples collected during fermentation runs give high activity, whereas the control sample (G-PA0804) yields a much lower value, often as little as 5% of the recombinant strains. Thus, this assay can differentiate recombinant hSOD activity from other SOD-like activities in the lysate and, as shown in Table 7, with a deviation generally less than 10%.

Table 7

SOD Assay Variation

Assay Sample (Run #-hours)	Activity[1] (units/μl)	% Deviation[2]
441-21	.791	10.44
441-54	1.779	4.00
442-21	.385	6.94
442-54	.569	11.40
445-34	.340	6.89
445-143	.442	6.85
804 (control)	.089	17.10

[1] Numbers represent the mean of 4 assay values.
[2] Calculated from the sample standard deviation of the mean.

The nitrite assay was standardized to the cytochrome c assay by testing both a human erythrocyte SOD standard (Sigma Chemical Company) and human recombinant SOD purified from P. pastoris in each of the assays. The erythrocyte SOD was reported by the manufacturer to have a specific activity of 2.7 units/μg in the cytochrome c assay. We have measured a specific activity of 3.8 units/μg in this assay and found that the purified recombinant hSOD prepared according to the invention, yielded the same value. All protein concentrations were verified using quantitative amino acid analysis. The nitrite assay also gave identical values for the two preparations. Therefore, the simpler and more discriminating nitrite assay can be used to assay lysate samples if the values obtained from unknowns are always compared with those obtained from pure enzyme of known specific activity.

Purification and stability of hSOD produced in P. pastoris

Greater than 90% of the recombinant hSOD was extracted from P. pastoris cells lysed with glass beads in the presence of simple buffers. Typically, a 50 mM

sodium or potassium phosphate buffer containing 0.1 mM EDTA at pH 7.8 was used, and recovery, after corrections for pellet volume, was at least 95 % when measured by either activity assay or Western blots. (Any buffer in which the enzyme is stable may be used.) Recombinant hSOD was purified from the cell lysate essentially as described by McCord et al., J. Biol. Chem. 241, 6049 (1969) for erythrocyte lysates.

Hemoglobin is precipitated from the erythrocyte preparation by an ethanol-chloroform treatment. Likewise, many yeast proteins were removed from the *P. pastoris* lysate. Ethanol, 25 % of the lysate volume, was added dropwise to the stirred lysate suspension that was kept below 4°C in a salt water-ice bath. Chloroform was added in a similar manner (15 percent of the initial lysate volume). Contaminating proteins were allowed to denature and precipitate by stirring for an additional 15 minutes in the ice bath, and the precipitate was removed by centrifugation.

Superoxide dismutase was further purified by a phase extraction step. Dibasic potassium phosphate was added to the supernatant that was warmed to 25°C. When thirty percent K_2HPO_4 (weight-to-volume) was dissolved in the ethanol-chloroform solution, hSOD partitioned into the upper phase away from the lower, high salt phase. The upper phase was cooled to 4°C and clarified by centrifugation.

Recombinant hSOD was concentrated by precipitating with cold acetone (75% of the supernatant volume), centrifuged, and resuspended in a reduced volume. The solution was dialyzed to equilibrium against 2.5 mM potassium phosphate, pH 7.8. (In place of potassium phosphate buffer any low ionic strength buffer around a neutral pH could be used.) This buffer should be the equilibration buffer for DE-52, or any other similar anion exchanger used in the next step.

hSOD eluted from DE-52 in a gradient of 2.5 to 100 mM potassium phosphate, pH 7.8.

When stored as a lysate at 3-5°C, the hSOD activity is stable for at least four weeks. As a purified protein, recombinant hSOD from P. pastoris is stable for at least eight weeks at 3-5°C.

Deposit

Viable cultures of P. pastoris strain GS115 were deposited, under the terms of the Budapest Treaty on the Deposit of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated thereunder, at the American Type Culture Collection, Rockville, Maryland, USA ("ATCC") on August 15, 1987 and were assigned ATCC Accession No. 20864.

The foregoing description details more specific methods that can be employed to practice the present invention. However detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

CLAIMS:

1. A method of increasing the production of a recombinant gene product from a culture of a recombinant methylotrophic yeast host, wherein said product is made by expression from a recombinant gene sequence operably associated with a methanol-responsive expression control element, which method comprises culturing said methylotrophic yeast host using a mixed nutriment feed, cell growth-gene induction mode comprising:

(a) a high growth phase wherein the nutriment medium contains a high concentration of multi-carbon, carbon-source nutriment with little or no methanol, for a period of time sufficient to in- crease the density of the viable yeast cells in the growth medium, without their producing by expression any substantial amount of said recombinant gene product,

(b) feeding a limiting amount of multi-carbon, carbon-source nutriment for a period of time sufficient to derepress the methanol metabolic pathway of said yeast host,

(c) allowing the fermentation culture to be maintained in a phase of high production of recombinant gene product during which time the methanol concentration is increased while maintaining a low multi-carbon, carbon-source nutriment.

2. The method according to Claim 1 wherein said phase (c) is conducted using a ratio of about 2:1 glycerol:methanol.

3. The method according to Claim 1 wherein said phase (c) is conducted using a ratio of about 4:1 glycerol:methanol.

4. The method according to Claim 1 wherein said phase (c) is conducted by adjusting the methanol concentration over time to provide a residual methanol concentration of up to about 1%.

5. The method according to any one of Claims 1, 2, 3, and 4 wherein said culturing is conducted at about 27 to 35 °C.

6. The method according to any one of Claims 1, 2, 3, 4, and 5 wherein said methylotrophic yeast host is a _Pichia_ _pastoris_ strain and said methanol-responsive expression control element is the AOX1 promoter.

7. The method according to any one of Claims 1, 2, 3, 4, 5, and 6 wherein said methylotrophic yeast host is a Mut^- strain.

8. The method according to any one of Claims 1, 2, 3, 4, 5, and 6 wherein said methylotrophic yeast host is a Mut^+ strain.

9. The method according to any one of Claims 7 and 8 wherein the recombinant expression product is an animal lysozyme c.

10. The method according to Claim 9 wherein the recombinant expression product is bovine lysozyme c2.

11. The method according to Claim 9 wherein the recombinant expression product is human lysozyme.

12. The method according to any one of Claims 7 and 8 wherein the recombinant expression product is selected from the group consisting of human EGF(1 - 52) and human EGF(1 - 48).

13. The method according to Claim 12 wherein the recombinant expression product is human EGF(1-48).

14. The method according to any one of Claims 7 and 8 wherein the recombinant expression product is human superoxide dismutase.

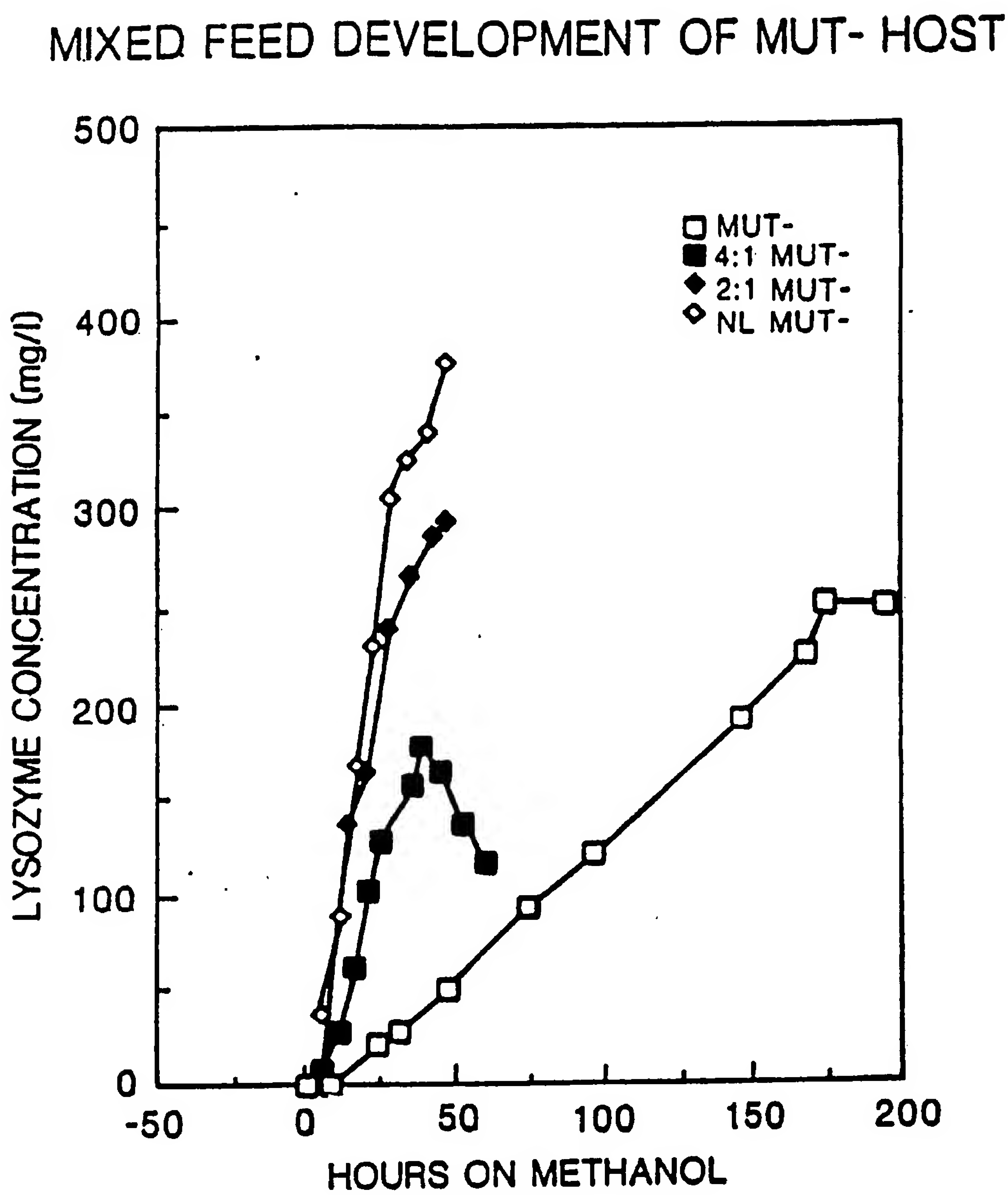
MIXED FEED DEVELOPMENT OF MUT- HOST
LYSOZYME CONCENTRATION (mg/l)
500
400
300
200
100
0
-50
0
50
100
150
200
HOURS ON METHANOL
MUT-
4:1 MUT-
2:1 MUT-
NL MUT-

FIG. 1

1 LITER BOVINE LYSOZYME FERMENTATIONS

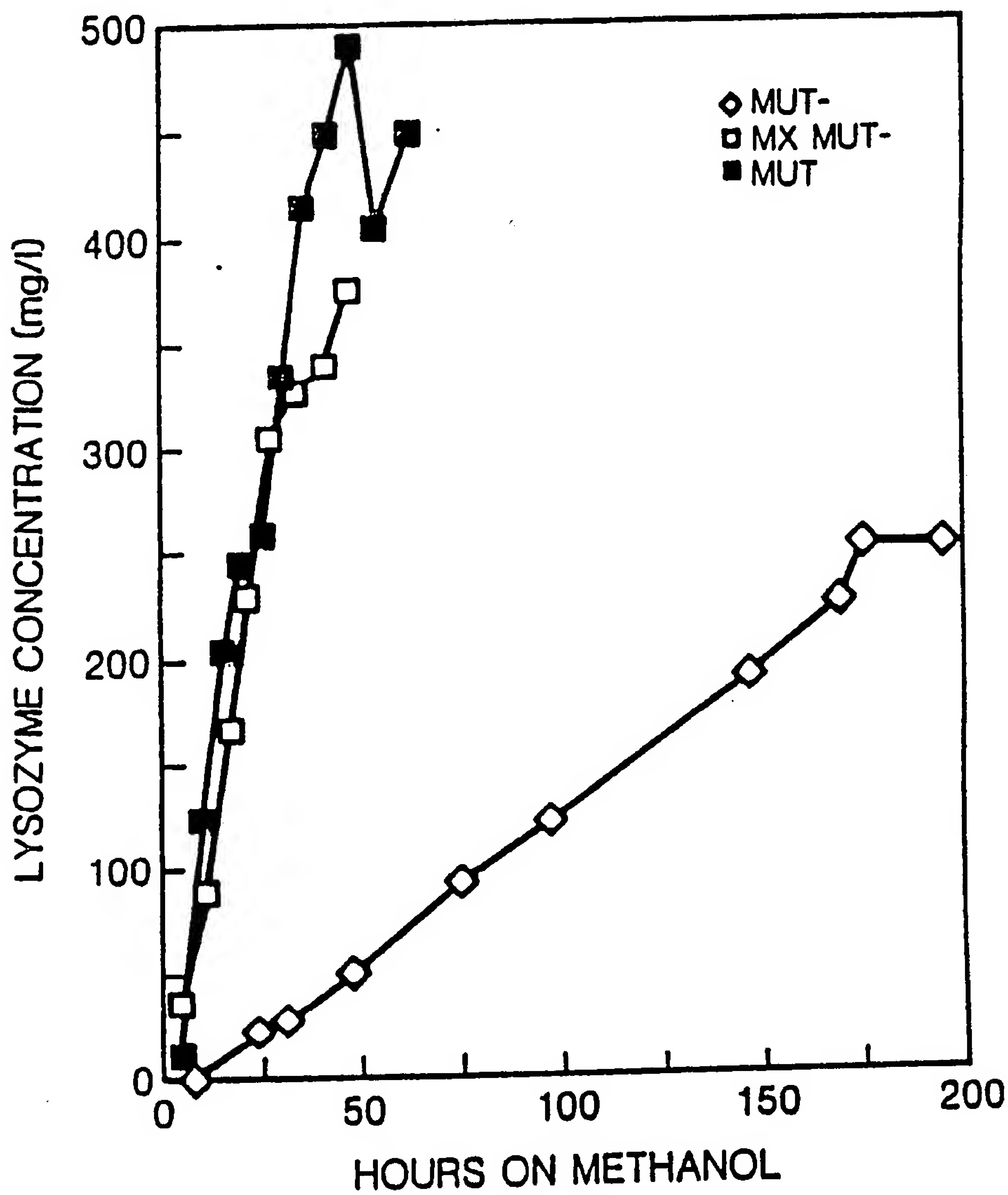

FIG. 2

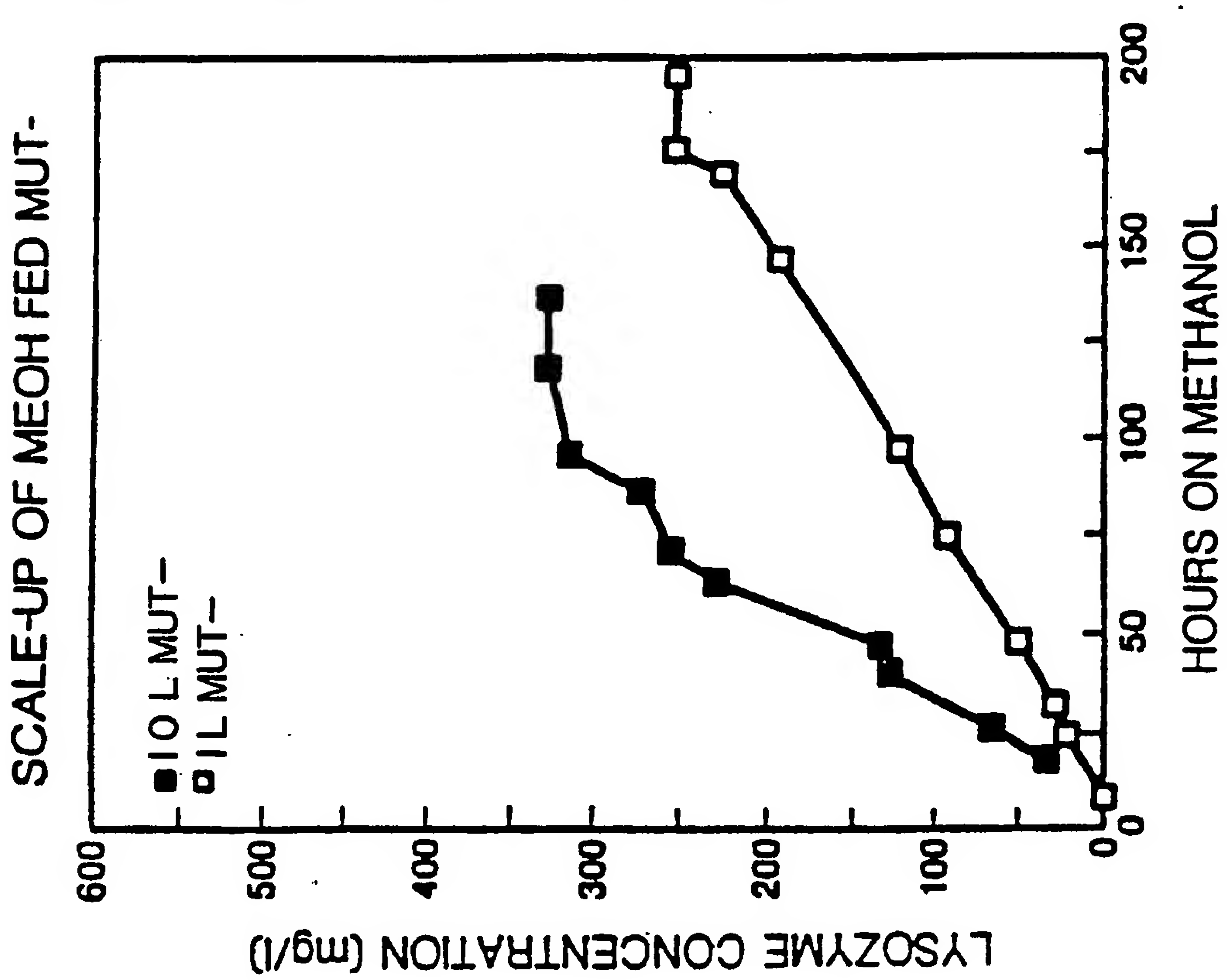

FIG. 3A

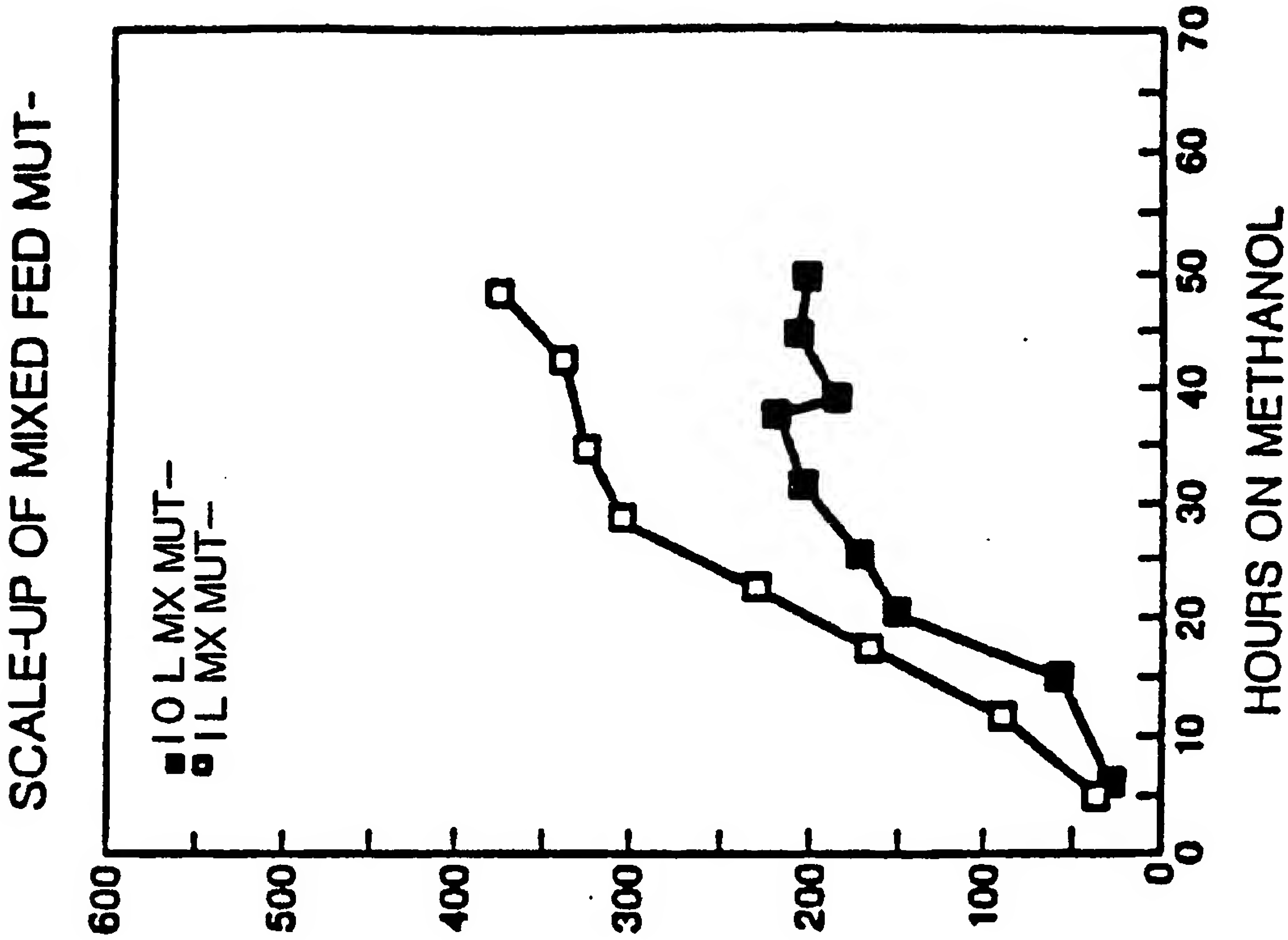

FIG. 3B

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04164

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C 12 N 5/00, 15/00; C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/ 68,172.3, 255

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

DIALOG: Biotech file 1969-1989; CAS File 1969-1989

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X / Y	Bio/Technology, Volume 5, published 05 May 1987. J.M. Cregg, et al. "High-level expression and efficient assebly of hepatitis B surface antigen in the methylotrophic yeast, Pichia pastoris" pp. 479-485. see entire article	1,2 / 3-14
Y	Microbial Growth C1 Compounds, Proc Int. Symp. (5th), published 1986. G. Thill, et al. "The methyltrophic yeast Pichia pastoris as a host for heterologous protein production" pp. 289-296. see entire article	1-14
Y	J. Biol. Chem., Volume 259, number 18, published 25 September 1984. P. Jolles, et al. "Stomach Lysozymes of ruminants" pp. 11617-11625. see entire article.	9,10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 December 1989	20 DEC 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Beth A. Burrous

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Proc. Natl. Acad Sci. USA, Volume 78, number 11, published November 1981. S. Suggs, et al. "Use of synthetic oligonucleotides as hybridization probes: isolation of cloned DNA sequences for human beta-2-microglobulin" pp. 6613-6617. see entire article.	9,10
Y	Gene, Volume 56, published May 1987. T. Hayakawa, et al. "Expression of human lysozyme in an insoluble form in yeast" pp. 53-59. see entire article.	11
Y	Proc. Natl Acad. Sci. USA, Volume 80, published December 1983. M.S. Urdea, et al. "Chemical synthesis of a gene for human epidermal growth factor urgosterone and its expression in yeast" pp. 7461-7465. see entire article.	12,13
Y	EP, A, 0 138 111 (Hallewell) 24 April 1985. see entire document.	14